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Research article

# Efficient expression and characterization of a cold-active endo-1, 4-β-glucanase from *Citrobacter farmeri* by co-expression of *Myxococcus xanthus* protein S

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ABSTRACT

*Background:* Cold-active endo-1, 4-β-glucanase (EglC) can decrease energy costs and prevent product denaturation in biotechnological processes. However, the nature EglC from *C. farmeri* A1 showed very low activity (800 U/L). In an attempt to increase its expression level, *C. farmeri* EglC was expressed in *Escherichia coli* as an N-terminal fusion to protein S (ProS) from *Myxococcus xanthus.*
*Results:* A novel expression vector, pET(ProS-EglC), was successfully constructed for the expression of *C. farmeri* EglC in *E. coli*. SDS-PAGE showed that the recombinant protein (ProS-EglC) was approximately 60 kDa. The activity of ProS-EglC was 12,400 U/L, which was considerably higher than that of the nature EglC (800 U/L). ProS-EglC was active at pH 6.5–pH 8.0, with optimum activity at pH 7.0. The recombinant protein was stable at pH 3.5–pH 6.5 for 30 min. The optimal temperature for activity of ProS-EglC was 30°C–40°C. It showed greater than 50% of maximum activity even at 5°C, indicating that the ProS-EglC is a cold-active enzyme. Its activity was increased by $Co^{2+}$ and $Fe^{2+}$, but decreased by $Cd^{2+}$, $Zn^{2+}$, $Li^{+}$, methanol, Triton-X-100, acetonitrile, Tween 80, and SDS.
*Conclusions:* The ProS-EglC is promising in application of various biotechnological processes because of its cold-active characterizations. This study also suggests a useful strategy for the expression of foreign proteins in *E. coli* using a ProS tag.



## 1. Introduction

Cellulose (the major component of biomass) is a widely available renewable resource [1,2]. The main component of cellulose is high molecular-weight polysaccharides composed of β-1, 4-linked glucose units. Endo-β-1, 4-glucanase (EglC, EC 3.2.1.4) is an important enzyme in cellulose degradation that randomly hydrolyzes the β-1, 4-glycosidic linkages within cellulose chains and releases smaller fragments of different lengths [3,4,5]. Endoglucanases are attracting increasing attention for their potential applications in the feed, detergent, silage, food, and textile industries [6,7]. Compared with mesophilic proteases, endoglucanases with low temperature activity can decrease energy costs and avoid product denaturation in biotechnological processes [8,9]. Although many endo-β-1, 4-glucanases from different microorganisms have been isolated and purified, the research on cold-active EglCs is currently lacking [10].

The microbial communities in the intestinal tracts of termites include a number of microbes that efficiently degrade cellulose [11,12,13]. In a previous study, a psychrophilic EglC was isolated from *Citrobacter farmeri* A1 in the wood-inhabiting termite *Reticulitermes labralis* (Unpublished observations). However, the activity of the *C. farmeri* EglC was very low. Therefore, in the present study, we developed an *Escherichia coli* expression system to increase its production level for analyzing enzymatic properties.

Two major roadblocks for expression of heterologous protein in *E. coli* are poor production and the formation of insoluble protein [14,15]. When the *EglC* gene was cloned into pET-32a and transformed into *E. coli*, it was very poorly expressed (unpublished observations). One approach to overcome this problem is to fuse with another protein, which can enhance the solubility of

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heterologous proteins expressed in *E. coli*. The protein S (ProS) tag from *M. xanthus* was reported to increase the solubility of target proteins, and importantly, the fusion did not affect the properties of the target protein [16,17]. For this strategy, the Trx tag in pET-32a was replaced with the ProS tag using cloning technique. Then, the *EglC* gene from *C. farmeri* A1 was ligated into the novel vector pET-ProS and expressed in *E. coli*. The recombinant ProS-EglC protein was also fully characterized.

## 2. Materials and methods

### 2.1. Strains and plasmids

In our previous study, the *EglC* gene was amplified from the *C. farmeri* A1 genome and deposited into the GenBank database (accession no. KT313000). The pMD20-T cloning vector was purchased from TaKaRa (China). *E. coli* DH5α and BL21 cells were obtained from our laboratory stocks. The pET-32a expression vector was purchased from Novagen (Germany).

### 2.2. Construction of pET-ProS vector

The pET-32a plasmid was used as the backbone to construct an expression vector containing a ProS tag (Fig. 1a). The sequence of *ProS* gene (the N-terminal domain of protein S; amino acid residues 1–92; GenBank accession no. J01745.1) from *M. xanthus* was obtained according to the previous paper [16,17,18,19,20]. The *ProS* gene was synthesized by Shanghai Geneary Biotech Co., Ltd. (China) with *Nde*I sites at both terminus. The Trx tag in pET-32a was removed by digestion with *Nde*I. Then, the purified *ProS* gene and pET-32a (without the Trx-tag) were ligated with T4 DNA ligase (TaKaRa, China) to generate the novel expression vector pET-ProS (Fig. 1a).

### 2.3. Construction and transformation of pET(ProS-EglC) vector

The mature DNA fragment of the *EglC* gene (without the signal peptide) was amplified by PCR with primers EglC-F (CTCCATGGGCCT GTACCTGGCCCGCAT) and EglC-R (CGCTCGAGATTTGAACTTGCGCAT TCCTG), which contain the *Nco*I and *Xho*I sites, respectively. The primers were designed based on the nucleotide sequence of *C. farmeri* A1 *EglC* (GenBank accession no. KT313000). The PCR program was as follows: 6 min at 95°C, 35 cycles of 40 s at 95°C, 55 s at 59°C, and 55 s at 72°C, and a final extension step for 8 min at 72°C. The purified PCR fragment was cloned into pMD20-T. Then, pMD20-EglC was double-digested with *Nco*I and *Xho*I, and the isolated *EglC* fragment was ligated into the pET-ProS vector. The obtained plasmid, pET(ProS-EglC), was transformed into *E. coli* BL21 to express recombinant ProS-EglC (Fig. 1a).

### 2.4. Expression and purification of the recombinant ProS-EglC

Recombinant *E. coli* pET(ProS-EglC) was grown at 37°C in 250-mL shaker flasks (at 200 rpm) containing LB medium. When the culture density reached an $OD_{600}$ of ~0.6, expression of the fusion protein was induced by isopropyl-1-thio-β-D-galactopyranoside (final concentration of 1 mM). After the cells were grown for 4 h, the culture cells were collected by centrifugation. The pellets were resuspended in 10 mL of $Na_2HPO_4$-citric-acid buffer (pH 7.0), and the cells were lysed by ultrasonication on ice.

The recombinant fusion protein was purified by 6×His-Tagged Protein Purification Kit (CWBIO, China). Briefly, the cell lysates containing His-tagged ProS-EglC was passed through the $Ni^{2+}$ affinity chromatography and washed by Soluble Binding Buffer (20 mM Tris–HCl with pH 7.9, 10 mM imidazole and 0.5 M NaCl). The bound proteins were eluted by Soluble Elution Buffer (20 mM Tris–HCl with pH 7.9, 500 mM imidazole and 0.5 M NaCl). The expressed proteins were characterized by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

### 2.5. Assay of ProS-EglC activity

ProS-EglC activity was measured by the DNS method. The standard reaction mixture, which consisted of 1 mL CMC-Na buffer (substrate) and diluted enzyme solution (1 mL), was heated at 40°C for 30 min in a thermostatic water bath. DNS (3 mL) was added to stop the reaction. The reaction was boiled for 5 min, and the amount of reducing sugars was assessed by measuring the absorbance at 540 nm using a spectrophotometer (Shanghai Precision & Scientific Instruments Co., Ltd., China). One unit of endoglucanase activity was defined as the amount of enzyme that produced 1 μmol of glucose per min under the assay conditions.

### 2.6. Biochemical characterization of ProS-EglC

To determine the optimal pH for ProS-EglC, the protein was incubated in buffers with various pH values ($Na_2HPO_4$-citric acid buffer, pH 3.5-pH 7.0 and sodium phosphate buffer, pH 8.0) at 40°C for 30 min. To measure pH stability, ProS-EglC was pre-incubated in buffers with different pH values (pH 3.5, 4.5, 5.5, and 6.5) at 40°C for 30 min. Then, residual activity was measured under optimal conditions (40°C, pH 7.0).

The optimum temperature for ProS-EglC activity was determined by incubating the reaction mixture at different temperatures (5°C–70°C)

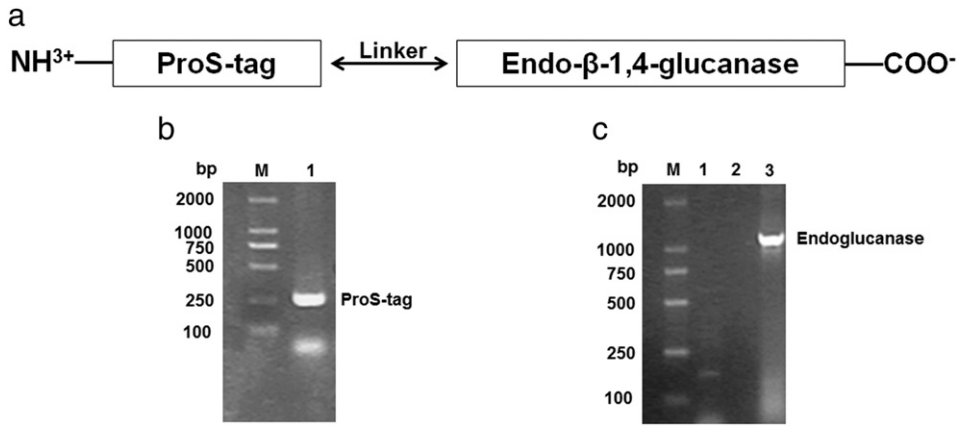


**Fig. 1.** Construction of expression vector pET(ProS-EglC). (a) Schematic presentation of a protein S fusion protein. (b) Amplification of protein S. Lane M: DNA markers (100–2000 bp); Lane 1: Protein S fragment. (c) Amplification of *EglC* gene. Lane M: DNA markers (100–2000 bp); Lanes 1–2: Negative control; Lane 3: *EglC* gene.

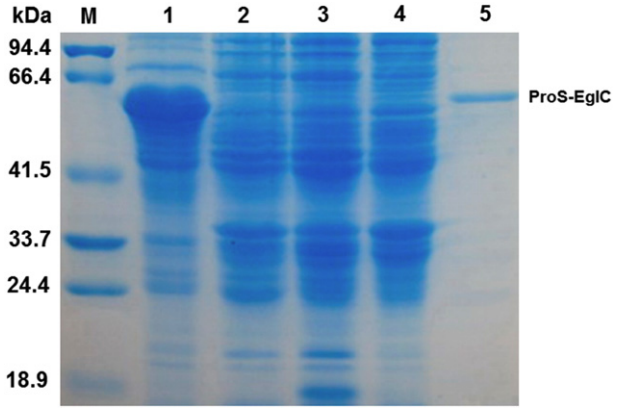


**Fig. 2.** SDS-PAGE analysis. Lane M: protein markers; Lane 1: IPTG induced *E. coli* pET(ProS-EglC); Lane 2: *E. coli* pET(ProS-EglC); Lane 3: IPTG induced *E. coli* pET-ProS; Lane 4: *E. coli* pET-ProS; Lane 5: Purified ProS-EglC.

at the optimal pH value (7.0). Thermal stability was evaluated by pre-incubation of samples without substrate at various temperatures (40°C–60°C) for 30 min, and then the enzyme samples were assayed for residual activity under standard assay conditions.

To determine the effect of metal ions and chemical reagents on ProS-EglC activity, various metal ions ($CoCl_2$, $FeCl_2$, $CdCl_2$, $ZnCl_2$, and $Li_2SO_4$,) and chemicals (methanol, Triton-X-100, acetonitrile, Tween 80, and SDS) were added to the reaction mixture. Then, the activities of ProS-EglC were measured under standard assay conditions. The reaction mixture without any ions or chemical reagents was considered as the control.

All the above measurements were carried out in duplicate. The statistical analyses of the values were performed by Microsoft Excel 2010. Data were presented as means with standard deviation (SD).

## 3. Results and discussion

### 3.1. Construction of the novel expression plasmid pET(ProS-EglC)

To improve its expression level, a ProS tag was fused to the N-terminus of *EglC* in an *E. coli* expression system (Fig. 1a). The novel expression plasmid pET(ProS-EglC) was obtained using vector pET-32a as the backbone. PCR analysis of pET(ProS-EglC) is shown in Fig. 1b and Fig. 1c. The sizes of the DNA fragments were similar to the expected sizes of *ProS* (276 bp) and *EglC* (1056 bp), suggesting that *ProS* and *EglC* genes were successfully cloned into the expression vector.

### 3.2. Expression and purification of ProS-EglC

SDS-PAGE was performed to analyze the expression products from *E. coli* pET(ProS-EglC). As shown in Fig. 2, crude protein extracts from the recombinant *E. coli* strain showed a strong band with a molecular weight (MW) of ~60 kDa, which was higher than the predicted MW of full-length EglC because of the fusion tag. However, this band was not present in the supernatant of the cell lysates from an uninduced recombinant strain (Fig. 2). Recombinant ProS-EglC was purified by affinity chromatography, since the SDS-PAGE analysis showed only one band (Fig. 2). The specific activity of purified ProS-EglC was 15.6 U/mg.

When the EglC gene from *C. farmeri* A1 was cloned into the pET-32a expression vector and transformed into *E. coli*, it was poorly expressed (data not shown). Therefore, the novel expression vector pET(ProS-EglC) was developed to increase its production level. In the present study, the cellular extract from *E. coli* pET(ProS-EglC) showed activity of 12,400 U/L, which was higher than that of the original endoglucanase from *C. farmeri* A1 (800 U/L). In addition, the ProS-EglC activity was also higher than that of the recombinant endoglucanase expressed in *E. coli* EF-EG2 (1000 U/L), *E. coli* Cel5D (1.44 U/mg), and *E. coli* Cel8H (4.9 U/mg) [21,22,23]. These results suggested that the presence of ProS tag in the N-terminus would make the foreign protein in a soluble fraction and dramatically increase expression level [17,18]. Furthermore, the recombinant protein fused with ProS tag maintains their functions [19,20].

### 3.3. Effect of pH on the recombinant ProS-EglC activity

The pH characteristics of recombinant ProS-EglC were similar to those of other neutral endoglucanases [24,25]. ProS-EglC was active at pH 6.5-pH 8.0, with optimum activity at pH 7.0 (Fig. 3a). At the pH values below 4.5, the activity of ProS-EglC rapidly decreased (Fig. 3a). ProS-EglC was stable and maintained greater than 70% of maximum activity after incubation at pH 3.5-pH 6.5 for 30 min (Fig. 3b).

### 3.4. Effect of temperature on the recombinant ProS-EglC activity

Many endo-β-glucanases from various microorganisms (*i.e.*, bacteria, fungi, and actinomycetes) have been studied [26]. However, only a few cold-active endoglucanases have been cloned and expressed

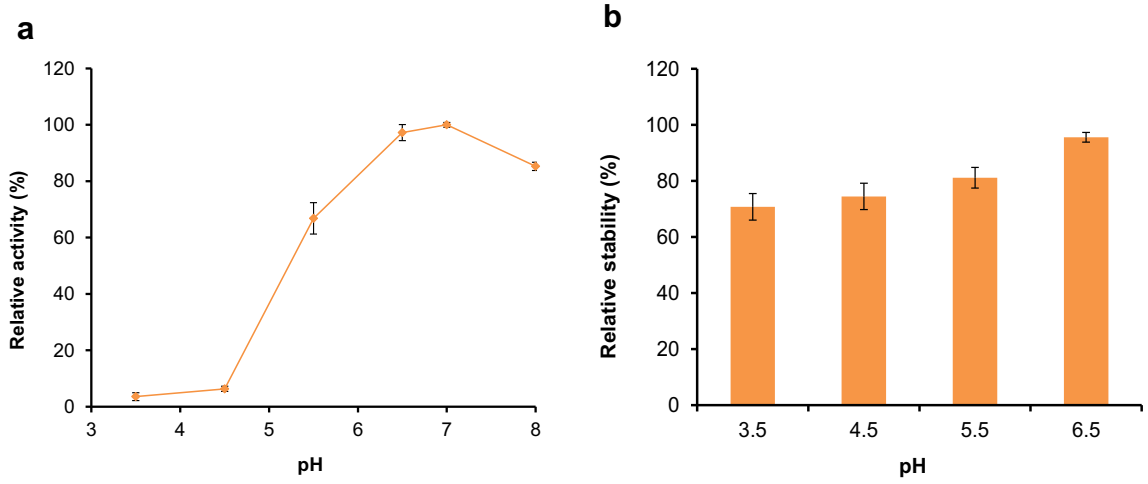


**Fig. 3.** Effect of pH on ProS-EglC activity and stability. (a) Effect of pH on the ProS-EglC activity. (b) The pH stability of ProS-EglC. The ProS-EglC activity determined at the optimal pH (7.0) and temperature (40°C) was considered as the control. Data were presented as means with SD.

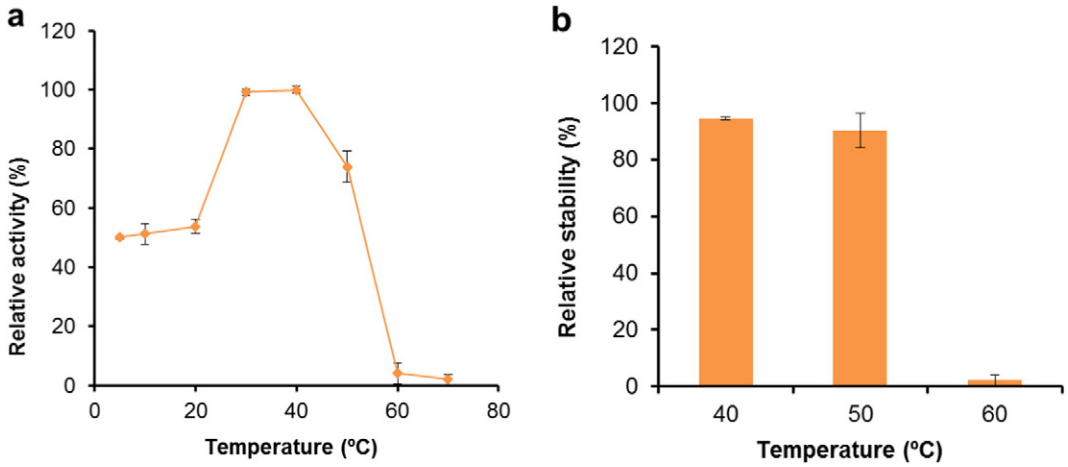


**Fig. 4.** Effect of temperature on ProS-EglC activity and stability. (a) Effect of temperature on the ProS-EglC activity. (b) The temperature stability of ProS-EglC. The ProS-EglC activity determined at the optimal pH (7.0) and temperature (40°C) was considered as the control. Data were presented as means with SD.

for specific applications [27]. Compared with mesophilic enzymes, cold-active endoglucanases have the advantage of avoiding alteration or denaturation of the product in biotechnological processes [21]. The typical properties of cold-active enzymes are relatively high activity at low temperatures and poor thermostability [28,29].

Fig. 4a showed that the optimal temperature of ProS-EglC was 30°C– 40°C, and the enzyme showed greater than 50% of maximum activity even at 5°C (Fig. 4a). In contrast, the activity of ProS-EglC decreased dramatically at the temperatures above 60°C (Fig. 4a). The results of the temperature stability assay showed that approximately 92% of ProS-EglC activity was lost after incubation at 60°C for 30 min (Fig. 4b). Based on these properties, the recombinant ProS-EglC protein expressed in *E. coli* pET(ProS-EglC) cells was a cold-active endoglucanase. Similar results have been reported for other low-temperature glucanases and cellulases from *Paenibacillus* sp. IHB B 3084 and *Eisenia fetida* [21,29]. However, the activities of general and thermostable endoglucanases are rapidly lost at the temperatures below 20°C [26,30].

### *3.5. Effects of chemical reagents and metal ions on the activity of ProS-EglC*

Martin et al. [31] and Rawat et al. [32] reported that $Co^{2+}$ and $Fe^{2+}$ enhanced the activity of endoglucanases from *E. coli* Rosetta 2 and *Aspergillus niger*, respectively. As shown in Fig. 5, the activity of ProS-EglC was increased by $Co^{2+}$ and $Fe^{2+}$. However, its activity was decreased by $Cd^{2+}$, $Zn^{2+}$, $Li^{+}$, methanol, Triton-X-100, acetonitrile, Tween 80, and SDS (Fig. 5). These inhibitive effects may be caused by denaturation of the protein structure [31].

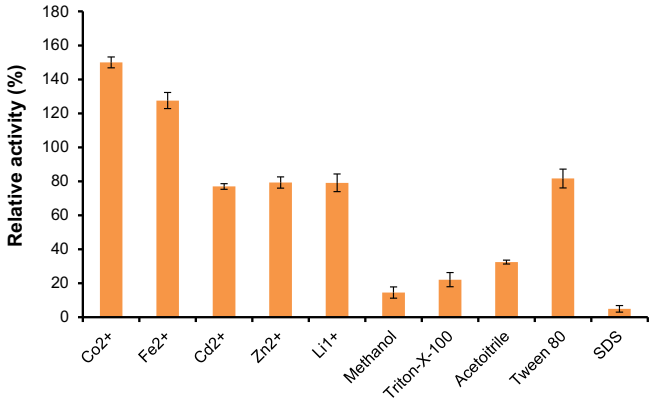


**Fig. 5.** Effects of chemical reagents and metal ions on the activity of ProS-EglC. The reaction mixture without any ions or chemical reagents was considered as the control. Data were presented as means with SD.

## 4. Conclusions

The main *EglC* gene from *C. farmeri* was successfully expressed in *E. coli* as a fusion with ProS tag. Based on characterizations, ProS-EglC is a typical cold-active enzyme. It has potential applications in numerous biotechnological processes. This study also suggests a useful strategy for improving heterologous protein expression in *E. coli*.

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## Conflict of interest

The authors confirm that this article content has no conflict of interest.

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